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Citation for published version:

French, CE, Nicklin, S & Bruce, NC 1996, 'Sequence and properties of pentaerythritol tetranitrate reductase from *Enterobacter cloacae* PB2', *Journal of Bacteriology*, vol. 178, no. 22, pp. 6623-6627.
<<http://jb.asm.org/content/178/22/6623.abstract>>

Link:

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Document Version:

Publisher's PDF, also known as Version of record

Published In:

Journal of Bacteriology

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Sequence and Properties of Pentaerythritol Tetranitrate Reductase from *Enterobacter cloacae* PB2

CHRISTOPHER E. FRENCH,¹ STEPHEN NICKLIN,² AND NEIL C. BRUCE^{1*}

Institute of Biotechnology, University of Cambridge, Cambridge CB2 1QT,¹ and E.S.C. Department, D.E.R.A., Fort Halstead, Sevenoaks, Kent TN14 7BP,² United Kingdom

Received 24 June 1996/Accepted 10 September 1996

Pentaerythritol tetranitrate reductase, which reductively liberates nitrite from nitrate esters, is related to old yellow enzyme. Pentaerythritol tetranitrate reductase follows a ping-pong mechanism with competitive substrate inhibition by NADPH, is strongly inhibited by steroids, and is capable of reducing the unsaturated bond of 2-cyclohexen-1-one.

We previously reported the isolation of *Enterobacter cloacae* PB2 on the basis of its ability to use nitrate esters such as pentaerythritol tetranitrate (PETN) and glycerol trinitrate (GTN) as nitrogen sources. *E. cloacae* PB2 possesses a soluble PETN reductase capable of reductively liberating nitrite from nitrate esters with oxidation of NADPH (1) (Fig. 1). PETN reductase is a monomeric flavoprotein of M_r 40 000. Recently, White et al. (20) reported the isolation of a strain of *Agrobacterium radiobacter* capable of growth with GTN as the sole nitrogen source and showed that cell extracts from this organism liberated nitrite from GTN and PETN with oxidation of NADH, suggesting the activity of a similar enzyme.

Nitrate esters, though produced in large amounts for use as explosives and vasodilators (13), are rare in nature (7, 12), and multiply substituted nitrate esters are not known to occur naturally. The origin of enzymes apparently specialized for their breakdown is therefore of interest. To investigate this question, the structural gene encoding PETN reductase, designated *onr* (for organic nitrate reductase), was cloned using degenerate oligonucleotide probes.

Cloning and sequence analysis of *onr*. The N-terminal sequence of PETN reductase purified from *E. cloacae* PB2 as previously described (1) was found to be SAEKLFPLKV GAVTAPNRVFMPLT. On the basis of *E. cloacae* typical codon usage, the following oligonucleotide probes were designed, based on residues 2 to 11 and 18 to 26, respectively: (i) AC-TTT-(G/C)AG-(G/C)GG-(G/C)GT-GAA-(G/C)AG-TTT-TTC-(G/C)GC; (iii) GT-(G/C)AG-(G/C)GG-(G/C)GC-CAT-GAA-(G/C)AC-(G/C)CG-GTT. Southern blots using standard procedures (15) showed that both probes bound to the same region of PB2 genomic DNA. A 1,525-bp *NcoI*-*ClaI* genomic DNA fragment was cloned in pBluescript SK+ (Stratagene) to give pONR1. Sequencing indicated the presence of an open reading frame beginning with codons matching the known N-terminal sequence of PETN reductase. The sequence of *onr* and deduced amino acid sequence of PETN reductase are shown in Fig. 2. The sequence predicts a protein of 364 residues with an M_r of 39,358 excluding the N-terminal methionine, consistent with the M_r of approximately 40,000 estimated for PETN reductase by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (1). A putative ribosome-binding site and transcription termination sequence

were detected. No obvious σ^{70} -like promoter sequence was present, but three regions between a putative upstream termination sequence and the ribosome-binding site have significant homology to *Escherichia coli* σ^S -dependent promoters (21).

The deduced amino acid sequence of PETN reductase was compared to sequences in protein and nucleic acid databases using the BLAST program of the GCG package (6). The most similar proteins found were members of the old yellow enzyme family of α/β -barrel flavoprotein oxidoreductases (17). These include old yellow enzyme of *Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae* (11, 14, 18), homologs from the yeast *Kluyveromyces lactis* (10) and the protozoan *Trypanosoma cruzi* (OWL:U31282), a steroid-binding protein from the yeast *Candida albicans* (9), and morphinone reductase from the bacterium *Pseudomonas putida* M10 (4, 5). Expressed sequence tags from the plants *Arabidopsis thaliana*, *Oryza sativa*, and *Brassica campestris* suggest the presence of a related enzyme in plants. The most similar enzyme is morphinone reductase of *Pseudomonas putida* M10, the only other known bacterial member of this subgroup. PETN reductase and morphinone

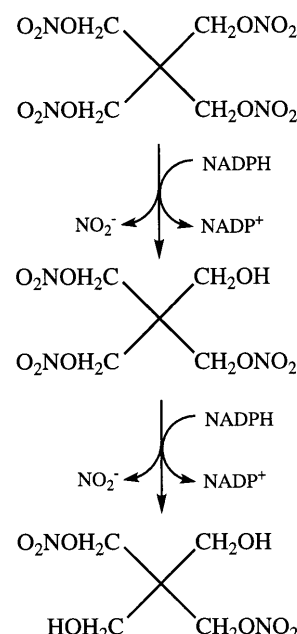


FIG. 1. Transformation of PETN by PETN reductase.

* Corresponding author. Mailing address: Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT, United Kingdom. Phone: 44-1223-334168. Fax: 44-1223-334162. Electronic mail address: N.Bruce@biotech.cam.ac.uk.

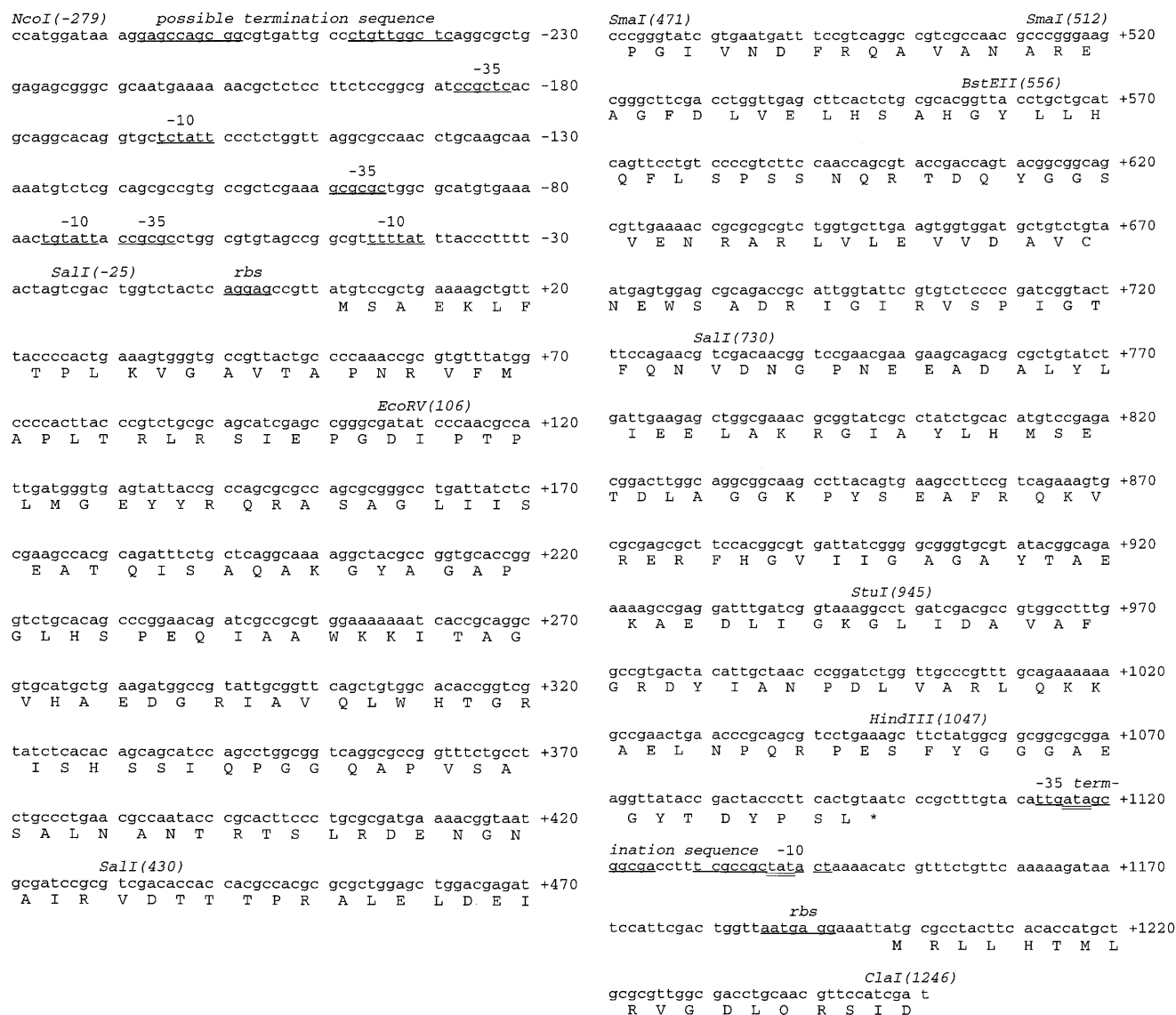


FIG. 2. Sequence of *onr* and deduced amino acid sequence of PETN reductase. Nucleotides are numbered with 1 being the A of the ATG initiating the gene. Putative ribosome-binding sites (*rbs*), transcription termination sequences, and the -35 and -10 regions of possible promoters are indicated.

reductase share 53% sequence identity and 71% sequence similarity as determined by the GAP program of the GCG package. An alignment of the deduced amino acid sequences of PETN reductase, morphinone reductase, and old yellow enzyme is shown in Fig. 3.

The physical properties of PETN reductase are consistent with a close relationship to the old yellow enzyme family. Like all of the known members of this group, it is a simple flavoprotein with flavin mononucleotide as a prosthetic group, its subunit M_r is approximately 40,000, and it is an oxidoreductase using a reduced pyridine nucleotide cofactor as an electron donor. However, all of these enzymes are active as homodimers, whereas the elution position of PETN reductase in gel filtration, as well as its significant passage through ultrafiltration membranes with a nominal M_r cutoff of 10,000 under high-salt conditions, suggest that PETN reductase is monomeric (1). Since PETN reductase is retained by ultrafiltration membranes under low-salt conditions, it is conceivable that

dimers form under such conditions; this possibility is under investigation.

The structure of old yellow enzyme has been shown to be an eight-stranded α/β -barrel (3). The flavin is hydrogen bonded by side chains from residues T-37, Q-114, R-243, and R-348 (3). All of these residues are conserved in both morphinone reductase and PETN reductase (Fig. 3). Of the residues which hydrogen bond with the flavin through the peptide backbone in old yellow enzyme, G-324 and G-347 are conserved, while several others are replaced by conservative substitutions such as glycine to alanine. Residues F-374 and Y-375, which provide a hydrophobic pocket for the dimethyl-benzene ring of the flavin, are also conserved, although F-296 is not.

Overexpression and purification of recombinant PETN reductase. The insert of pONR-1 is immediately downstream of the *lac* promoter of pBluescript SK+. It was found that *E. coli* JM109/pONR-1 grown in a rich medium such as SOB (15) in the presence of 0.4 mM isopropyl- β -D-thiogalactopyr-

MorB	1	PDT SFSNPGLFTP	LQLGSLSLPN	RVIMAPLT..	RSRTPDSVPG	41
Onr	1	SAEKLFTP	LKVGAVTAPN	RVFMAPLTRL	RSIEPGDIPT	38
OYE1	1	SFVKDFKPQ	ALGDTNLFPK	IKIGNNELH	RAVIPPLTRM	49
			SA	SB	S1	
MorB	42	R.LQQIYYGQ	RA..SAGLII	SEATNISPTA	RGYVYTPGIW	88
Onr	39	P.LMGEYYRQ	RA..SAGLII	SEATQISAQA	KGYAGAPGLH	85
OYE1	50	RDWAVEYYTQ	RAQRPGTMII	TEGAFISQA	GGYDNAPGVW	99
		H1		S2		H2
MorB	89	VVEAVHAKGG	RIALQLWHVG	RVS.HELVQP	DGQQPVAPSA	137
Onr	86	ITAGVHAEDG	RIAVQLWHTG	RIS.HSSIQP	GGQAPVSASA	134
OYE1	100	IFNAIEKKKS	FVWVOLWVLG	WAAFPDNLAR	DGLRYDSASD	142
			S3	HA	SC	
MorB	138	EFEDGTAGLH	PTSTP.RALE	TDEIPGIVED	YRQAAQRAKR	186
Onr	135	D.ENGNAIRV	DTTTP.RALE	LDEIPGIVND	FRQAVANARE	182
OYE1	143	MDAEQEAkak	KANNPOHSLT	KDEIKOYIKE	YVOAAKNSIA	192
		HB	SD	H3		S4
MorB	187	ANACLPNQFL	ATGTNRRTDQ	YGGSIENRAR	FPLEVVDAVA	236
Onr	183	AHGYYLLHQFL	SPSSNQRTDQ	YGGSVENRAR	LVLEVVDVAV	232
OYE1	193	ANGYLLNQFL	DPHSNTRTDE	YGGSIENRAR	FTLEVVDALV	242
		HC		H4		S5
MorB	237	RLTPFLELFG	LTDD.EPEAM	AF..YLAGEL	DRRG....L	278
Onr	233	RVSPIGTFQN	VDNGPNNEAD	AL..YLIEEL	AKRG....I	275
OYE1	243	RLSPYGVFNS	MSGGAETGIV	AOYAVVAGEL	EKRAKAGKRL	292
			H5			S6
MorB	279IGGDI	TYPEGFREQM	RQRFKGGLIY	CGNYDAGRAQ	323
Onr	276AGGK.	PYSEAFRQKV	RERFHGVIIG	AGAYTAEKAE	319
OYE1	293	TNPFLTEGEG	EYEGGSNDFV	YSIWKGPIVR	AGNFALHP..	339
			H6	S7		H7
MorB	324	...VAFGRP	FIANPDLPER	FRLGAALNEP	DPSTFYGGAE	369
Onr	320	...VAFGRD	YIANPDLVAR	LQKKAELNPQ	RPESFYGGGA	364
OYE1	340	KRTLIGYGRF	FISNPDLVDR	LEKGLPLNKY	DRDTFYQMSA	389
		S8	HD	H8		HE
MorB	370	NGHDRLG				376
Onr						
OYE1	390	EALKLGDWKK				399

FIG. 3. Alignment of the deduced amino acid sequences of PETN reductase (Onr), morphinone reductase (MorB), and old yellow enzyme (OYE1). The alignment was generated by the PILEUP program of the GCG package. Structural elements from OYE1 shown are taken from reference 6. The α -helices and β -strands forming the eight-stranded α/β -barrel are shown as H1 to H8 and S1 to S8, respectively; extra-barrel helices are shown as HA to HE, and extra-barrel strands are shown as SA to SD.

anoside (IPTG) expressed high levels of PETN reductase activity, suggesting that PETN reductase formed 30 to 50% of soluble cell protein. Assays of enzyme activity were performed as described previously (1). In the absence of IPTG, expression was approximately half of that observed with IPTG, suggesting that expression is driven both by the *lac* promoter of the vector and by a promoter in the insert. Recombinant PETN reductase was purified by affinity chromatography. Cells from 1 liter of stationary-phase culture of *E. coli* JM109/pONR1 grown in SOB medium were disrupted using a French press, and clarified extract was loaded onto a column with a 5-cm diameter packed with 70 ml of Mimetic Orange 2 A6XL (Affinity Chromatography Ltd., Freeport, Ballasalla, Isle of Man, United Kingdom) (packed height 3.6 cm) at a flow rate of 4 ml/min. The column was washed with 300 ml of 10 mM phosphate buffer, pH 7.0. Bound PETN reductase was then eluted with 60 mM ammonium acetate. The most active fractions, totalling 60 ml, were pooled and freeze-dried. The freeze-dried powder was stored at -20°C and made up as required in 50 mM phosphate buffer, pH 7.0. The crude extract contained 2,250 U

of activity at a specific activity of 6.1 U/mg, and the product contained 1,100 U at 13.6 U/mg, a yield of 49% at a purification factor of 2.2. The purified enzyme appeared homogeneous by SDS-PAGE and comigrated with PETN reductase purified from *E. cloacae* PB2. The N-terminal 10 amino acid residues of the recombinant enzyme were determined and were found to be identical to those of the enzyme purified from *E. cloacae* PB2.

Kinetic mechanism. To investigate the kinetic mechanism of PETN reductase, activities were measured at various concentrations of GTN and NADPH. GTN was used as a substrate rather than PETN because its greater solubility allows variation of the substrate concentration over a much wider range. GTN concentration was varied between 10 and 100 μM and NADPH concentration was varied between 40 and 150 μM . In all cases, GTN was added from a 1,000 \times stock solution in ethanol. Addition of ethanol at several times this level had no effect on the observed rate. Apparent V_{max} and K_m values were determined at each NADPH concentration by nonlinear regression using the GraFit software package (8). Figure 4A

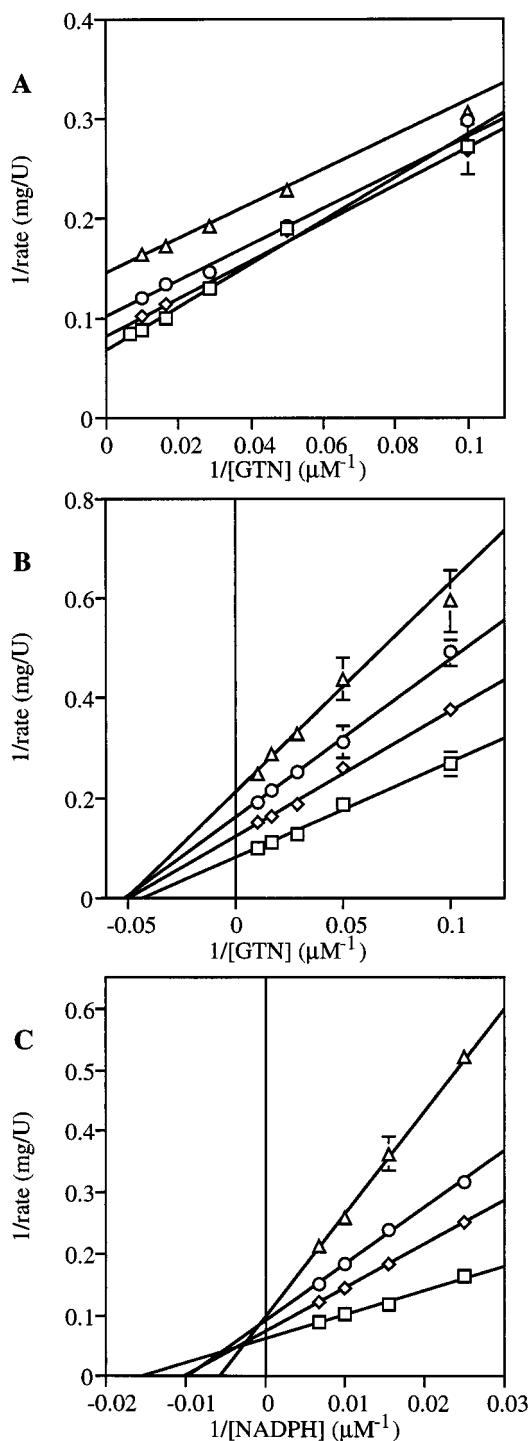


FIG. 4. (A) Double-reciprocal plot showing PETN reductase activity at various levels of GTN and NADPH. Lines shown were fitted separately to each data set by nonlinear regression using the GraFit software package (8). Each point shown is the mean of three independent assays. Error bars indicate one standard error. Triangles, 40 μM NADPH; circles, 65 μM NADPH; diamonds, 100 μM NADPH; squares, 150 μM NADPH. (B and C) Double-reciprocal plots showing inhibition of PETN reductase activity by testosterone. (B) NADPH concentration fixed at 100 μM and GTN concentration varied between 10 and 100 μM . Squares, no inhibitor; diamonds, 0.05 μM testosterone; circles, 0.1 μM testosterone; triangles, 0.2 μM testosterone. (C) GTN concentration fixed at 100 μM and NADPH concentration varied between 40 and 150 μM . Squares, no inhibitor; diamonds, 0.05 μM testosterone; circles, 0.1 μM testosterone; triangles, 0.2 μM testosterone.

shows a double-reciprocal plot including the lines fitted by nonlinear regression. Convergence occurs to the right of the y axis. This is characteristic of a ping-pong mechanism with competitive inhibition by the fixed substrate, in this case NADPH (2). In physical terms this implies that NADPH is capable of binding unproductively to the reduced form of the enzyme. The general equation for such behavior is

$$v = \frac{V_{\max}[A][B]}{[A][B] + K_B[A](1 + [A]/K_{AR}) + K_A[B]}$$

where $[A]$ is the concentration of NADPH, $[B]$ is the concentration of GTN, v is the observed rate, V_{\max} is the theoretical rate at saturating substrate concentrations ignoring substrate inhibition, K_A is the apparent K_m for NADPH at saturating levels of GTN, K_B is the apparent K_m for GTN at saturating levels of NADPH and K_{AR} is the dissociation constant for unproductive binding of NADPH to the reduced form of the enzyme.

Parameters for this equation were estimated by linear regression analysis of the slopes and intercepts of the lines shown in Fig. 4A. The following values were obtained: $V_{\max} = 25.2 \pm 1.5$ U/mg; $k_{\text{cat}} = 16.6 \pm 1.0$ s $^{-1}$; $K_A = 107 \pm 10$ μM ; $K_B = 39.3 \pm 3.5$ μM ; and $K_{AR} = 400 \pm 58$ μM . Errors indicated are one standard error based on the error of the linear regression.

A partial F-test was performed to determine whether the substrate inhibition term was statistically significant in describing the data. The F statistic was calculated as 30.1 with 1 numerator and 68 denominator degrees of freedom. This value is significant at the 1% level of confidence.

Similar kinetic behavior has been described for morphinone reductase, in which substrate inhibition was observed at high levels of NADH, and product inhibition patterns also suggested that the reduced product hydrocodone could bind unproductively to the reduced form of the enzyme (4, 5).

It has been found that NADP(H) is bound in an unusual manner in old yellow enzyme, such that the adenine portion is unbound and binding is primarily due to the nicotinamide ring and the nicotinamide ribose moiety (3). In this regard it is interesting that PETN reductase, like old yellow enzyme, is NADPH-dependent, whereas morphinone reductase, which shares 53% sequence identity with PETN reductase, is NADH dependent and does not appear to be significantly active with NADPH (4). In studies of the crystal structure of old yellow enzyme, it was necessary to use an NADP analog, since oxidized cofactor does not bind to oxidized enzyme, nor does reduced cofactor bind to reduced enzyme (3); by contrast, the reduced forms of both morphinone reductase and PETN reductase appear to show significant binding to the respective reduced cofactors. Further study of these enzymes may therefore cast light upon the unusual mode of cofactor binding in this growing enzyme family.

Interaction with steroids. Old yellow enzyme, estrogen-binding protein and, morphinone reductase are all known to bind steroids and to reduce the double bond of 2-cyclohexen-1-one (4, 9, 11, 16, 19). Several steroids were tested as inhibitors of PETN reductase and were found to be potent inhibitors of GTN reduction. The concentrations of progesterone, testosterone, and cortisone required to halve the observed rate at 100 μM GTN and 100 μM NADPH were approximately 0.022, 0.12, and 0.31 μM , respectively.

Inhibition by testosterone was investigated in more detail. Assays were performed with testosterone concentrations ranging from 0.05 to 0.20 μM , either with NADPH fixed at 100 μM and GTN varied between 10 μM and 100 μM or with GTN fixed at 100 μM and NADPH varied between 40 μM and 150

TABLE 1. Apparent kinetic parameters for substrates of PETN reductase^a

Substrate	K_m (mM)	V_{\max} (U/mg)	k_{cat} (min ⁻¹)	k_{cat}/K_m (min ⁻¹ mM ⁻¹)
GTN	0.023 ± 0.002	12.1 ± 0.5	480 ± 20	21,000 ± 3,000
EGDN ^b	2.4 ± 0.3	9.5 ± 0.5	380 ± 20	160 ± 30
2-cyclohexen-1-one	0.74 ± 0.06	3.2 ± 0.1	125 ± 3	170 ± 20

^a Apparent kinetic parameters were measured at 0.1 mM NADPH. Kinetic parameters were estimated by nonlinear regression using the GraFit software package (8). Errors shown are one standard error.

^bEGDN, ethylene glycol dinitrate.

μM. Apparent V_{\max} and K_m for each testosterone concentration were determined by nonlinear regression using the GraFit software package (8). Double-reciprocal plots showing data and fitted lines are shown in Fig. 4B and C. Convergence to the left of the y axis occurs with both GTN and NADPH as varied substrates, implying that testosterone binds to both the oxidized and the reduced forms of the enzyme. The general equation describing this behavior is

$$v = \frac{V_{\max}[A][B]}{[A][B] + K_B[A](1 + [A]/K_{AR} + [I]/K_{IR}) + K_A[B](1 + [I]/K_{IO})}$$

where K_{IO} is the dissociation constant for the binding of the inhibitor to the oxidized form of the enzyme, K_{IR} is the dissociation constant for the binding of the inhibitor to the reduced form of the enzyme, and other parameters are as previously described.

Using the values for V_{\max} , K_A , K_B , and K_{FA} determined previously, K_{IO} and K_{IR} were estimated from linear slope replots of the lines fitted for each inhibitor concentration. K_{IO} was estimated as 70 ± 6 nM and K_{IR} was estimated as 136 ± 7 nM. Errors indicated are one standard error based on the error of the linear regression in each case. By contrast, apparent K_m values for GTN were in the order of 10 to 30 μM, depending on the concentration of NADPH.

The α/β-unsaturated ketones 2-cyclohexen-1-one and codeinone were investigated as substrates for PETN reductase. Activity was detected with 2-cyclohexen-1-one. Apparent kinetic parameters measured in the presence of 0.1 mM NADPH are shown in Table 1, together with the corresponding values for the known substrates GTN and ethylene glycol dinitrate. Kinetic parameters for PETN could not be estimated due to its poor solubility in aqueous solutions (1). No activity was detected with codeinone, the best known substrate for morphinone reductase.

The observations that both morphinone reductase and PETN reductase bind steroids more strongly than their known substrates and that they are most closely related to eukaryotic enzymes suggest that they may be descended from a eukaryotic steroid reductase.

Nucleotide sequence accession number. The nucleotide sequence of *onr* has been submitted to GenBank and has been assigned the accession number U68759.

PETN, GTN and EGDN were kindly provided by the Defense Research Agency (Fort Halstead, Kent, United Kingdom).

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